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Abstract

The interest in perivascular cells as a niche for adult hematopoietic stem cells (HSCs) is significantly growing. In the adult bone marrow (BM), perivascular cells and HSCs cohabit. Among perivascular cells, pericytes are precursors of mesenchymal stem/stromal cells (MSCs) that are capable of differentiating into osteoblasts, adipocytes and chondrocytes. *In situ,* pericytes are recognised by their localisation to the abluminal side of the blood vessel wall, closely associated with endothelial cells in combination with the expression of markers such as CD146, neural glial 2 (NG2), platelet derived growth factor receptor β (PDGFRβ), α-smooth muscle actin (α-SMA), nestin (Nes) and/or leptin receptor (LepR). However, not all pericytes share a common phenotype: different immunophenotypes can be associated with distinct mesenchymal features, including hematopoietic support. In adult BM, arteriolar and sinusoidal pericytes control HSC behaviour, maintenance, quiescence and trafficking through paracrine effects. Different groups identified and characterized hematopoietic supportive pericyte subpopulations using various markers and mouse models. In this review, we summarize recent work performed by others to understand the role of the perivascular niche in the biology of HSCs in adults, as well as their importance in the development of therapies.

Key words (max 6 words): HSC, pericytes, niche, CD146, nestin, LepR

Running title Pericytes, niche for HSCs

ABBREVIATIONS

α Smooth Muscle Actin (α-SMA)

Acute Myeloid Leukaemia (AML)

Adventitial Reticular Cells (ARC)

Bone Marrow (BM)

Colony forming unit-fibroblasts (CFU-F)

C-X-C Motif Chemokine Ligand 12 (Cxcl12)

Cxcl12-abundant reticular (CAR) cells

Diphtheria toxin receptor (DTR)

Fluorescent activated cell sorting (FACS)

Hematopoietic stem cells (HSCs)

Hematopoietic stem/progenitor cell (HSPCs)

Leptin Receptor (LepR)

Mesenchymal stem/stromal cells (MSCs)

Nestin (Nes)

Neural glial 2 (NG2)

Perivascular Cells (PVCs)

Platelet derived growth factor Receptor (PDGFR)

Stem cell factor (Scf)

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1. INTRODUCTION

Adult hematopoietic stem cells (HSCs) are a rare subpopulation of cells residing in the bone marrow (BM) with a well-defined phenotype, multi-lineage developmental potential, the capacity to reconstitute irradiated host recipients over the long-term *in vivo,* and the capacity to self-renew as shown by serial transplantations (Kaimakis et al., 2013; Bhattacharya et al., 2016). In healthy subjects, HSC maintenance, behaviour and trafficking is largely dependent upon information they receive from the surrounding microenvironment or niche in which they are embedded. The concept of the niche was initially suggested after the work of Schofield in 1978 (Schofield, 1978) and has been defined as a small functional compartment with a specific anatomical position within an organ that homes and regulates stem cell activity, quiescence, self-renewal and differentiation for healthy tissue maintenance and repair (Mercier et al., 2012; Scadden, 2006; Watt & Hogan, 2000).

The hematopoietic niche of the BM is very complex and highly heterogeneous. It includes non-hematopoietic cell types such as osteoblasts, endothelial cells, pericytes, adipocytes, Schwann cells and nerves, and other hematopoietic cell types such as macrophages, osteoclasts, megakaryocytes, lymphocytes and neutrophils (Birbrair & Frenette, 2016). Recently, the putative existence of several niches for distinct haematopoietic outputs has been suggested (Mercier et al., 2012), due to variations in the composition and location of the immediate niche. Understanding how the different cell types are involved in the haematopoietic niches and whether and how the niches interact with each other and with HSCs is key to understand how the HSCs are regulated in homeostasis and hence, how they can be controlled in disease.

Mesenchymal stem/stromal cells (MSCs) were first described by Friedenstein (Friedenstein et al., 1978) and coined later on by Caplan in 1991 (Caplan, 1991). MSCs support haematopoietic stem cells in long term cultures *in vitro* via cell contact and secreted factors (Gottschling et al., 2007; Sorrentino et al., 2008; Van Overstraeten-Schlogel et al., 2015), and enable HSC engraftment and repopulation when co-

transplanted in both murine recipients and human patients (Battiwalla & Hematti, 2009; Jui et al., 2012; Le Blanc et al., 2007). However, MSCs were only characterised in culture retrospectively using the minimal criteria defined by the International Society for Cellular Therapy in 2006: MSCs adhere to plastic culture dishes, express CD90, CD105, CD73 and CD44, do not express endothelial and hematopoietic markers CD45, CD34, CD14, CD11b, CD79α, CD19 and HLA-DR, and differentiate *in vitro* into osteoblasts, adipocytes and chondrocytes (Dominici et al., 2006). Nevertheless MSCs remain heterogeneous and thus interest in prospectively isolating them directly from organs before culture has been continuously growing. Common mesenchymal/stromal markers associated with haematopoiesis are Stro-1, CD271, CD146, stem cell factor (Scf), Nestin (Nes), Sca-1, C-X-C motif chemokine ligand 12 (CXCL12), Leptin Receptor (LepR), Alkaline Phosphatase and CD105 (Flores-Figueroa et al., 2012). Importantly, recent studies have demonstrated that *in vivo*, such MSCs reside in the perivascular wall of the blood vessels in multiple organs (Corselli et al., 2012; Crisan et al., 2008; Murray et al., 2014) and are actively present in the haematopoietic niche (Birbrair & Frenette, 2016; Morrison & Scadden, 2014).

This review focuses on the role of different subsets of pericytes found to be integral components of adult haematopoietic stem cell niches, and highlights their importance as targets in developing new strategies to control disease progression.

2. PERICYTES, PRECURSORS OF MESENCHYMAL STEM/STROMAL CELLS

2.1. Definition

Pericytes are perivascular stellate cells that form a discontinuous layer in close contact with the endothelial cells surrounding capillaries and a continuous layer around microvessels (Diaz-Flores et al., 1991). They were first described as Rouget cells by Charles Marie Rouget (Rouget, 1873), but were renamed in 1923 by Zimmermann, as pericytes (Zimmermann, 1923). Early studies suggested vascular support as the major role of pericytes through direct cell contact and cell communication *via* specific junctions.

They are contractile and regulate vessel diameter (Gerhardt & Betsholtz, 2003) and maintain blood vessel integrity and permeability (Stallcup et al., 2016). More recently, stem cell properties have been associated with pericytes.

2.2 Pericytes share similar features with mesenchymal stem/stromal cells

Perivascular cells gained further interest when they were described to be *bona fide* MSCs. Present in virtually all vascularized organs, pericytes adhere to plastic culture dish, express MSCs markers CD90, CD44, CD105 and CD73 before and after culture and have adipogenic, osteogenic and chondrogenic potentials, cardinal properties of MSCs (Crisan et al., 2008; Crisan et al., 2011; Doherty et al., 1998; Farrington-Rock et al., 2004; Lin et al., 2008; Pierantozzi et al., 2015; Zimmerlin et al., 2014).

Recent studies have investigated pericyte involvement in bone and cartilage formation and regeneration, cardiac regeneration, brain vasculature, cancer angiogenesis, immunomodulation and hematopoietic support. Pericytes from multiple human organs regenerate damaged muscle fibres in different mouse models (Crisan et al., 2008; Dellavalle et al., 2011; Park et al., 2011) and improve cardiac function when injected into myocardial infarcted heart (Chen et al., 2013). However, cardiac derived pericytes are unable to differentiate into skeletal muscle fibres although they can differentiate into other mesodermal lineages – adipocytes, osteoblasts and chondrocytes- suggesting the existence of heterogeneity between pericytes from different organs (Chen et al., 2015). Pericytes derived from skeletal muscle, adipose tissue or BM successfully mineralise or regenerate the broken bone *in vivo* (Crisan et al., 2008; James et al., 2012a; James et al., 2012b; Sacchetti et al., 2007; Sacchetti et al., 2016). Importantly, pericytes associated with small vessels of the bone marrow and white adipose tissue, have been shown to support hematopoiesis *in vitro* and *in vivo* (Birbrair & Frenette, 2016; Corselli et al., 2013; Morrison & Scadden, 2014). Additionally, another perivascular cell type, adventitial pericytes, located in the adventitia layer of large blood vessels, was also described to display mesenchymal potential. $CD34^{+}CD31^{-}$ adventitial

pericytes can differentiate into mesodermal lineages, especially contributing to angiogenesis (Campagnolo, 2010; Zengin, 2006), and are suggested to be pericyte progenitors (Howson, 2005; Corselli, 2012).

In conclusion, pericytes are endowed with mesodermal differentiation potential when seeded in specific conditions *in vivo* and *in vitro,* and actively participate in the regeneration process under experimental conditions. It is important to understand their physiological roles in tissue development and homeostasis, and whether they directly participate in repair and regeneration processes. Some studies have started to address this using cell lineage tracing experiments and have shown that pericytes can naturally generate and/or regenerate certain tissues (Bouacida et al., 2012; Dulauroy et al., 2012; Feng et al., 2011; Goritz et al., 2011; Kozanoglu et al., 2009; Krautler et al., 2012; Olson & Soriano, 2011; Tang et al., 2008; Zhao et al., 2014). However, pericytes are heterogeneous and may fall into functionally distinct groups based on their location and on the pathological state of the organ (Birbrair et al., 2014a; Birbrair et al., 2014b; Birbrair et al., 2013a, Birbrair et al., 2013b; Birbrair et al., 2013c). Thus, clear identification and characterisation of different pericyte subsets *in situ* is necessary for further investigations.

2.3 Markers of perivascular cells

Most of the pericyte markers described so far in the literature are also present in other cell types. In addition, not all pericytes express the same markers. Therefore pericytes require a combination of markers for their proper characterisation. Here we summarize the most commonly used markers for pericytes in both human and mouse tissues.

Also known as M-CAM or MUC18, CD146 adhesion molecule is a membrane glycoprotein (Shih, 1999) that plays a role in the interaction between endothelial cells and pericytes, by binding to laminin α4 (Ishikawa et al., 2014). The germline CD146 knock-out mice are viable with no specific phenotype (Jouve et al., 2015). First identified in melanoma cells and associated with tumour metastasis and vascularization (Lehmann

et al., 1987), CD146 is also expressed by human pericytes and endothelial cells (Crisan et al., 2008; Sers, Riethmüller, & Johnson, 1994), smooth muscle cells (Sers et al., 1994; Tormin et al., 2011) and few T and B lymphocytes (Covas et al., 2008).

Nestin (Nes) is an intermediate filament protein type IV that binds to and regulates the assembly of intermediate filaments such as vimentin, desmin and internexin (Steinert et al., 1999). Germline deletion of *nestin* is embryonic lethal. Few mice survive and have impaired coordination suggesting a role in the neural system (Park et al., 2010). Indeed, nestin was described as a neural stem cell maker (Birbrair et al., 2013b; Lendahl, Zimmerman, & McKay, 1990) and was also detected in pericytes in the brain, skeletal muscle and BM (Alliot et al., 1999; Birbrair et al., 2013b; Mendez-Ferrer et al., 2010). Subsequently, nestin has also been described in cancer cells (Krupkova et al., 2010), myofibroblasts (Kishaba et al., 2010) and endothelial cells (Mokry et al., 2004; Suzuki et al., 2010). Not all pericytes express nestin. Pericytes of the blood vessels in the rat testis only express it transiently during the regeneration of chemoablated Leydig cells (Davidoff et al., 2004).

Leptin Receptor (LepR) also known as CD295 and OB-R, is a type I transmembrane glycoprotein class I cytokine receptor (Zhang et al., 1997). The ligand of LepR is leptin, an adipocyte-derived hormone firstly associated to lipid metabolism and obesity (Coleman, 1978). Germline LepR knock out mice are obese with hyperglycemia, glucose intolerance and elevated levels of insulin (Wang, Chandrasekera, & Pippin, 2014). Leptin/LepR signalling was shown to be involved in immune homeostasis, reproduction, haematopoiesis, angiogenesis and haematological malignancies (Han & Wang, 2015; Konopleva et al., 1999) and bone formation (Chen & Yang, 2015). In addition, LepR is expressed in pericytes surrounding sinusoids in the mouse BM and microvessels in human adipose tissue (Corselli et al., 2013; Ding et al., 2012; Zhou et al., 2014).

Other markers expressed by pericytes are neural glial antigen 2 (NG2), platelet derived growth factor receptors (PDGFRα, PDGFRβ) and α-smooth muscle actin

(αSMA). NG2, also known as chondroitin sulphate proteoglycan 4, plays a role in cell proliferation and migration during vasculogenesis, and binding to collagen α2 and β1 integrin (Stallcup & Huang, 2008). NG2 knock-out mice are not lethal and do not have an apparent phenotype (Grako et al, 1999). However, under a high fat diet, NG2 knock-out mice become obese and have fatty livers (Chang et al., 2012). Moreover, the permeability of the blood vessels increases with a decrease in pericyte coverage (Stallcup et al., 2016). This is in line with NG2 expression in pericytes of the arterial system (Crisan et al., 2009; Murfee et al, 2005). NG2 is also expressed on macrophages, glial cells and various tumour cells (Murfee et al., 2006). PDGFRα is a cell surface tyrosine kinase that interacts with PDGFA, PDGFB, integrin β3, caveolin-1 and nexin among other molecules (Ricono et al., 2009; Morikawa et al., 2009). PDGFRβ plays a key role during capillary sprouting recruiting pericytes *via* PDGFB/PDGFRβ signalling (Hellstrom et al., 1999; Stapor et al, 2014; Lindahl et al., 1997). PDGFRβ is also expressed on smooth muscle cells, interstitial fibroblast and glial cells (Bonner, 2004; Robbins et al., 1994). α-SMA is expressed in pericytes, smooth muscle cells and myofibroblasts (Cherng et al., 2008). Smooth muscle actin plays a role in cell motility and vessel integrity (Nehls & Drenckhahn, 1991). Not all pericytes express α-SMA and together with NG2, can be used to discriminate subsets of pericytes such as pericytes associated with arterioles ($NG2^{+}$α-SMA^{+}), capillaries ($NG2^{+}$α-SMA^{-}) and venules ($NG2^{-}$ α-SMA^{+}) (Nehls & Drenckhahn, 1991; Crisan et al., 2008, 2009).

In summary, pericytes express different markers that may vary between species, tissues or developmental stage. This phenotypic heterogeneity suggests that pericytes surrounding distinct blood vessel types may also play different functions.

3. PERICYTES, INTEGRAL COMPONENTS OF THE ADULT HEMATOPOIETIC STEM CELL NICHES

In the past, MSCs have increasingly been implicated in HSC support as major components of the HSC niche (Kfoury & Scadden, 2015). As pericytes were shown to be a reservoir of MSCs *in vivo* and in addition, proximal to HSCs, recent studies have focused on their role in HSC regulation. The BM is located in the bone cavity which is highly vascularised especially enriched in arterioles, thin-wall innervated vessels surrounded by smooth muscle cells, and sinusoids, more evenly distributed, larger and fenestrated that allows cell trafficking between the BM and the blood stream (Hooper et al., 2009; Itkin et al., 2016; Khan et al., 2016; Kunisaki et al., 2013). Both arteriolar and sinusoidal pericyte populations have been recently studied as part of the hematopoietic niches using distinct marker expression as for example CD146, Nes and LepR (Figure).

3.1 CD146 perivascular cells support the adult hematopoietic stem cells

In human adult BM, CD146 expression is found near sinusoids in subendothelial cells, also called adventitial reticular cells (ARCs). In 2007, the group of Paolo Bianco demonstrated that $CD146^+$ ARCs are key players in the niche of HSCs (Sacchetti et al., 2007). In this study, they showed that $CD146^+$ cells express known mesenchymal markers e.g. CD105, CD63, alkaline phosphatase, among others, and have the ability to form colony-forming unit-fibroblasts (CFU-F). They are in contact with endothelial cells and they express other pericyte markers such as αSMA, NG2 and PDGFRβ, and are negative for hematopoietic and endothelial markers (CD45, CD34 and CD31) (Sacchetti et al., 2007). Interestingly, when transplanted into immunodeficient mice, $CD146^+$ cells formed ossicles composed of donor derived bone and sinusoids in combination with host derived endothelium and hematopoietic cells demonstrating the ability of these prospectively purified cells to support haematopoiesis *in vivo.* Not all single $CD146^+$ clones were able to generate heterotopic ossicles following transplantation, suggesting that the $CD146^+$ population is heterogeneous or changes in culture. Indeed, in hypoxic

conditions, CD146 is downregulated in culture (Tormin et al., 2011). Furthermore, $CD146^{-/low}$ cells of the trabecular bone or periosteum are also able to form bone upon transplantation, but do not transfer the hematopoietic microenvironment, suggesting that not all bone progenitors support HSCs *in vivo* (Sacchetti et al., 2007). Cultured $CD146^{+}$ cells highly express osteogenic progenitor transcripts (*COL1A1*, *COL1A2*, *RUNX2*, etc.) and HSC niche related transcripts, such as Jagged-1 (Notch ligand), N-Cadherin, CXCL12 and the KIT ligand (SCF) (Sacchetti et al., 2007). These molecules have been implicated in the maintenance of HSCs in the mouse BM *in vitro* and *in vivo* (Duncan et al., 2005, Sugiyama et al., 2006, Wilson & Trumpp, 2006).

In addition to BM, $CD146^{+}$ pericytes purified from the human adult white adipose tissue are also able to support hematopoietic stem/progenitor cells (HSPCs) *ex vivo* (Corselli et al., 2013). This is not a surprise. In the mouse, white adipose tissue contains a population of immature HSCs that have a transcriptional profile very similar to BM HSCs (Poglio et al., 2012). A previous study showed that transplantation of the stromal vascular fraction (SVF) derived from murine adipose tissue containing stromal cells, adipocyte progenitors and hematopoietic progenitors ($CD45^{+}CD34^{+}$) into lethally irradiated immunodeficient mice, was able to rescue the host hematopoietic system (Cousin et al., 2003), suggesting that white adipose tissue contains stromal cells with hematopoietic supportive ability. *Corselli et al.* demonstrated that only the $CD146^{+}$ cell population supports HSC activity in co-culture experiments with human cord blood hematopoietic stem cells, which is in line with *Sacchetti et al.* observations (2007). Primary immunodeficient recipients were engrafted at long-term when injected with $CD45^{+}$ donor hematopoietic cells from $CD146^{+}$co-cultures and could further repopulate secondary recipients. $CD146^{+}$cells were able to activate Notch signalling in HSPCs (Corselli et al., 2013), in agreement with previous reports suggesting that Notch signalling regulates the growth and differentiation of HSPCs *via* the microenvironment (Bigas et al., 2010). Interestingly, $CD146^{+}$ pericytes from the fetal BM and adult adipose tissue co-express Nestin, CXCL-12 and LepR, markers that have previously been used

to define pericytes with HSC niche activity in the murine BM (Ding et al., 2012; Mendez-Ferrer et al., 2010; Sugiyama et al., 2006). In conclusion, $CD146^+$ perivascular cells support HSCs.

3.2 Nestin-expressing pericytes support hematopoietic stem cells and are highly heterogeneous

Pericytes expressing nestin ($Nestin^+CD31^-CD34^-VE\text{-}Cadherin^-CD45^-$) were identified and described in the BM of Nestin-GFP mice (Mendez-Ferrer et al., 2010). These cells were found within the BM parenchyma and in regions adjacent to the bone. Nes-GFP^+ (Nes^+) cells highly express HSC maintenance related genes such as Cxcl12, Adrb3 and Angiopoietin-1, and can be cultured as *mesenspheres* in non-adherent cultures that self-renew and differentiate into osteoblasts, adipocytes and chondrocytes (Mendez-Ferrer et al., 2010). *In vivo,* serial transplantations revealed that Nes^+ pericytes generate ectopic bone, self-renew and support hematopoiesis (Mendez-Ferrer et al., 2010). Similar to the mouse, human fetal and adult BM also contain a similar population of Nes^+ cells. However, this population is heterogeneous and can be further separated on the expression of CD105 (Isern et al., 2013), a cell surface marker of osteoprogenitor cells (Aslan et al., 2006). Furthermore, all CFU-F and sphere-forming capacities were observed in $CD105^+CD146^+$ cells but not in $CD105^+CD146^-$ nor $CD105^-CD146^-$ cells (Isern et al., 2013), in line with previous reports (Sacchetti et al., 2007; Tormin et al., 2011). In addition, $CD34^+$ human cord blood-derived hematopoietic cells expanded when co-cultured with these mesenspheres, were multilineage and serially engrafted immunodeficient recipients in transplantation assays suggesting that at least a subset of human Nes^+ cells also support HSCs (Isern et al., 2013).

To test the *in vivo* requirement of murine Nes^+ cells in HSC maintenance*, Mendez-Ferrer et al.,* (2010) further crossed a Cre-inducible diphteria toxin receptor (iDTR) mouse line with Nes-cre^{ERT2} mice. This model enables the selective deletion of Nes-cre^{ERT2} cells upon diphtheria toxin injection. Ablation of these cells led to a decrease

in the BM HSC number mainly due to their reduced homing abilities, suggesting that Nes^+ pericytes are involved in the regulation of HSC traffic. HSC number was proportionally increased in the spleen, but whether the HSCs found in the spleen were proximal to pericytes was not shown.

Another Nes^+ pericyte subset with MSC activity in the mouse BM was further identified (Pinho et al., 2013). $Nes^+CD51^+PDGFR\alpha^+$ triple positive cells show higher levels of *Cxcl12, Vcam1, Angpt1, Scf and Opn*, all HSC regulatory genes, compared to the $Nes^+CD51^-PDGFR\alpha^-$, $Nes^+CD51^+PDGFR\alpha^-$ and $Nes^+CD51^-PDGFR\alpha^+$ populations. These populations were clonally expanded as mesenspheres, and single spheres were implanted into ectopic grafts. Only triple positive cells were able to self-renew and transfer the hematopoietic microenvironment (Pinho et al., 2013). These cells largely overlap with $LepR^+$ perivascular cells, which are a major producer of SCF in the murine BM (Ding et al., 2012). Conditional deletion of *Scf* in Nes^+ cells (*Nes-cre/Scf$^{fl/-}$* or *Nes-creER/Scf$^{fl/-}$*) did not reduce HSC frequency in the BM, but instead, it induced a significant decline in HSCs in the spleen, suggesting that Nes^+ cells are also a component of the HSC niche in the spleen (Ding et al., 2012).

The maintenance of HSC numbers in Nes-cre/$Scf^{fl/-}$ mice or Nes-creER/$Scf^{fl/-}$ is in line with the lack of nestin expression found in Scf-GFP cells: while Nes-GFP^+ cells showed the same distribution as Scf-GFP^+ cells, $Cherry^+$ cells from Nes-Cherry/Nes-GFP double transgenic mice, as well as $EYFP^+$ cells from recombined Nes-cre/*loxP-EYFP* mice were only detected around large blood vessels in bone cryosections (Ding et al., 2012). *In situ* characterization of Nes^+ cells in the BM of wild-type mice will therefore be necessary to better understand and clarify the role of Nes^+ pericytes in the maintenance of HSCs in the BM.

Nes^+ cells occupying a perivascular position were confirmed in human fetal and adult BM (Ferraro et al., 2011; Pinho et al., 2013). In the fetal BM $Nes^+PDGFR\alpha^+CD51^+$ cells were detected in close contact with αSMA vasculature and HSPCs, and expression of high levels of HSC maintenance genes (*Cxcl12, Vcam1, Angpt1, Opn and Scf*) was

detected between 15 and 20 weeks of gestation. Interestingly, CD146 was homogeneously expressed in these cells, but only a small fraction of $CD146^+$pericytes expressed nestin, PDGFRα and CD51. This fraction was enriched in HSC niche transcripts and CFU-F activity compared to non-triple positive cells (Pinho et al., 2013), suggesting that the $CD146^+$ pericytes previously described in human tissues are heterogeneous (Corselli et al., 2013; Sacchetti et al., 2007). Transplantations of human $CD51^+PDGFR\alpha^+$ cells isolated from fetal BM into immunodeficient mice also led to the formation of HSC ectopic niche which recruited host HSCs to the grafts. In addition, mesenspheres cultured from this same population were able to expand HSPCs *ex vivo* prior engraftment into recipient mice (Pinho et al., 2013).

Kunisaki et al. further investigated the distribution of Nes-GFP in pericytes around arterioles and sinusoids based on transgene expression (Kunisaki et al., 2013). Nes-GFP^{bright} cells were rarer and found exclusively along arterioles in both sternal and long BM (Nes^{peri}) while Nes-GFP^{dim} were associated with sinusoids and displayed a more reticular shape (Nes^{retic}) (Kunisaki et al., 2013). Although both subpopulations were capable of CFU-F formation, most colonies were formed from Nes^{peri} cells. RNA sequencing data showed that the expression of HSC niche related genes *Cxcl12*, *Scf*, *Angtp1*, *Vcam1* and *Opn*, was higher in Nes^{peri} cells compared to Nes^{retic} cells. Nes^{retic} cells were found to largely overlap with the $LepR^+$ pericytes (major SCF producers) described by *Ding et al.* (Ding et al., 2012), while no overlap was detected between Nes^{peri} and $LepR^+$ cells (Kunisaki et al., 2013). In addition, Nes^{peri} but not Nes^{retic} cells expressed the pericytic markers αSMA and NG2. NG2 expression in the BM was restricted to approximately 30% of Nes^{peri} cells. Diphtheria toxin-induced depletion of $NG2^+$cells in NG2-cre^{ERTM}/iDTR recombined mice altered the localization of HSCs away from arterioles suggesting a role in HSC quiescence and maintenance (Kunisaki et al., 2013). In conclusion, nestin-expressing perivascular cells support HSCs and are highly heterogeneous.

3.3 Leptin Receptor- expressing cells secrete factors that are crucial for HSC maintenance

In a study performed to unravel how *Scf* expressing cells affect HSC maintenance in the mouse BM, Ding et al. observed a $LepR^+$ pericyte population that was restricted to Scf-GFP^+ cells in the mouse BM sinusoids (Ding et al., 2012). Gene expression profiling of $LepR^+$ pericytes showed expression of MSC genes such as *Cxcl12*, *alkaline phosphatase*, *PDGFRα and PDGFRβ,* with no nestin expression detected. Surprisingly, and in contrast with all the perivascular cell populations with MSC activity previously described, bone-lining cells from LepR-*cre*/*loxP*-EYFP mice did not express EYFP *in situ*, suggesting that $LepR^+$ cells do not give rise to osteoblasts *in vivo* (Ding et al., 2012). *Cxcl12* conditional deletion in LepR-*cre*;$Cxcl12^{fl/-}$ mice led to a reduction in the frequency of HSPCs in adult BM with an increase in their number in the circulation, suggesting a role for this chemokine in retaining HSCs in the BM niche (Ding & Morrison, 2013). Conditional deletion of *Scf* from Lepr-*cre* cells led to a reduction in HSC number in the BM, but not in the developing liver of newborns (Ding et al., 2012). Thus, $LepR^+$ pericytes play an important role in HSC maintenance in the mouse BM. In human fetal BM and adipose tissue, immunofluorescence showed no differences in LepR expression between $CD146^+$ and $CD146^-$ cultured cells (Corselli et al., 2013). It will be important to test whether similar results are observed *in situ* prior to culture, or if $CD146^-$ cells purified from these tissues upregulate LepR expression under the culture conditions used. Upon co-culture with $CD146^-$ cells, human cord blood derived $CD34^+$ hematopoietic cells did not engraft primary recipient mice *in vivo* (Corselli et al., 2013) raising the question as to whether in these human tissues, LepR specifically marks HSC niche cells at least in culture. $LepR^+$ pericytes were later shown to largely overlap with Nes-GFP^+ pericytes in LepR-*cre*/*LSL-tdtomato*/Nes-GFP mice (~80% of the cells), and 63% with the $Nes^+CD51^+PDGR\alpha^+$ population (Pinho et al., 2013). In conclusion, $LepR^+$ pericytes contribute to the niche of hematopoietic stem cells and overlap with other pericyte subsets described as integral part of the niche.

4. PERICYTES AS POTENTIAL TARGETS FOR THERAPY DEVELOPMENT

Changes in the hematopoietic niche have been implicated in diseases, such as leukemia. It has been long known that the microenvironment is a key player in many primary tumours as well as in metastasis. Drug development using cancer cells without taking into account their surroundings has enabled the treatment of certain diseases, but has proven to be insufficient. The development of more efficient therapies should take into account cells that surround and support malignant cells, i.e. the tumour microenvironment (McMillin et al., 2013).

Stromal cells can influence the response of cancer cells to chemotherapy. For instance, certain leukemic cells are more resistant to chemotherapeutic agents in the presence of the BM stromal cells than when cultured alone (Moshaver et al., 2008; Polak et al., 2015; Zhang et al., 2012). Interestingly, the opposite effect (known as synthetic lethality) has also been described, in which tumour cells become more susceptible to drugs within a certain microenvironment (McMillin et al., 2010). This resistance or susceptibility implies that bidirectional communication between leukemic cells and their microenvironment exists (McMillin et al., 2013). For instance, acute myeloid leukemia (AML) was shown to alter the hematopoietic niche (Hanoun et al., 2014). AML leads to a reduction in $NG2^+$ perivascular cell numbers and to denervation of arterioles. Due to the loss of catecholaminergic nerve fibres that are required to maintain the quiescence of MSCs (Mendez-Ferrer et al., 2008), Nes^+ pericytes are expanded and become more committed to the osteoblastic lineage that supports leukaemia progression. In addition, HSC maintenance gene expression is greatly reduced in these cells, leading to depletion of healthy HSCs in the BM (Hanoun et al., 2014). Another study showed that myelodysplastic cells in patients were able to reprogram MSCs to establish a stem cell disease niche unit (Medyouf et al., 2014). In contrast, initial perturbation of mouse osteoprogenitors was also able to induce BM dysfunction and myelodysplasia suggesting that the tumour microenvironment also plays a key role in cancer initiation

and progression (Raaijmakers, 2011; Raaijmakers et al., 2010). Overall it is crucial that pre-clinical studies include cells that constitute the microenvironment in order to have a more realistic idea of the possible outcomes of treatments *in vivo*.

Most studies focusing on the effects of the tumour microenvironment on cancer cells and their response to chemotherapy were performed using heterogeneous mesenchymal stromal cells. Understanding the mechanisms underlying drug resistance or sensitivity in more purified stromal cell populations, as well as how alterations to these cells are linked with disease is imperative for the development of novel and more precise treatments. Many chemotherapeutic agents target cycling cells, and therefore do not affect the most quiescent ones, which is one of the major causes of relapse. Pericytes in the BM niche were shown to maintain the quiescence of HSCs and protect them against genotoxic insults in the mouse (Kunisaki et al., 2013). In addition, depletion of these $NG2^+$ cells activated HSC cycling and mobilization (Kunisaki et al., 2013). Targeting these pericytes in leukemic patients could be a potential way of overcoming the quiescence leukemic stem cells and thus decreasing the chance of relapse.

Another important aspect to take into account when developing new strategies to treat tumours with high mortality rates is metastasis. A major driver of metastasis is angiogenesis. In cancer, the stable association between pericytes and endothelial cells is disrupted followed by changing in vascular permeability allowing cancer cells to enter the bloodstream. BM biopsies from patients with AML display enhanced angiogenesis at diagnosis. It was found that pericyte coverage is reduced compared to healthy BM. When remission is achieved, microvessel density is restored and pericyte counts increase (Weidenaar et al., 2011). Treatment with drugs that interfere with VEGF/VEGFR signalling were shown to induce a normalized vessel pattern and increase the pericyte coverage of the tumour blood vessels (Jain, 2005)(Jain, 2005). Another way to stimulate blood vessel coverage with pericytes was shown in hereditary haemorrhagic telangiectasia (HTT), where patients suffer from vascular malformations leading to frequent nosebleeds. Thalidomide was shown to reduce the severity and frequency of

the bleeding. In the mouse model for HTT, thalidomide treatment was shown to stimulate the pericyte coverage of blood vessels by increasing PDGFB expression in endothelial cells, which leads to the activation and recruitment of pericytes (Lebrin et al., 2010). Increased vascular permeability and decrease in HSC number in the bone marrow was also found in diabetes, in both patients and mouse models (Oikawa et al., 2010; Spinetti et al., 2013). Decrease in the density of microvessels including capillaries, sinusoids and arterioles, and functional alterations of endothelial cells were found. It would be thus important to investigate whether different pericyte subsets shown to have a role in the HSC niche are also affected. We can speculate that, in diabetes, some of these pericytes change their behaviour and/or differentiate, thus losing their HSC supportive roles. Indeed, higher adipose tissue deposition was found in the bone marrow of the diabetic mice but also in patients with type 2 diabetes (Spinetti et al., 2013). This may secondarily influence the cell cycle status of HSCs together with their mobilisation into the bloodstream or their survival as previously suggested (Mangialardi et al., 2014).

5. CONCLUSIONS

We have here reviewed the concept that within the perivascular component, different subtypes of HSC supportive cells exist. We know that these subtypes are distinct and to some degree overlapping (Figure, Table). However, some discrepancies exist between studies. This may be due to the technical approach, e.g. the experimental conditions employed, the mouse models used, or the recombination efficiency. For instance, Nes-GFP$^+$ cells from Nes-GFP mice show a very different distribution when compared to Nes-*cre* and Nes-*creER* cells, and, depending on the model, play distinct roles on HSC maintenance (Ding et al., 2012; Mendez-Ferrer et al., 2010). In addition, a perivascular cell population called CXCL12-abundant reticular (CAR) cells, has been described in a peri-sinusoidal position in CXCL12-GFP and CXCL12-DTR-GFP mice (Omatsu et al., 2010; Sugiyama et al., 2006). The number of HSCs as well as lymphoid

and myeloid progenitors were reduced two days after CAR cells were depleted upon diphtheria toxin injection. The remaining HSCs were more quiescent suggesting that CAR cells influence HSC activation in the mouse BM. Whether CAR cells overlap with Nes^+ or $LepR^+$ supportive pericytes remains unclear (Table).

Additionally, BM is highly complex and a variety of other niche components are involved in HSC support in addition to the perivascular cells. Whether and how these components interact with each other to regulate HSCs remains to be investigated. As the microenvironment is expected to maintain tissue homeostasis, it is reasonable to think that compensatory mechanisms take place when the niche is altered, as for example when a specific cell type is depleted. Studying all niche components simultaneously is challenging and to date none of the existing mouse models can be used to face this complexity. Currents models only allow study of perivascular cell subsets individually and thus studies should qualify their conclusions until further research should bring clarifications. Given that pericytes are involved in such a variety of pathologies, overcoming these technical limitations will allow a better understanding of their function in homeostasis and disease, and pave way for novel therapies.

More questions remain unanswered. Recent studies have shown that there are more than one type or subtype of HSCs in the BM (Benz et al., 2012; Challen et al., 2010; Crisan et al., 2015). Some are controlled by bone morphogenic protein (BMP) and others are regulated by the transforming growth factor β (TGFβ) signalling pathway. It is crucial to address whether these types or subtypes are proximal to a particular niche for a better understanding of the development of specific types of leukaemia and other diseases.

Conflict of Interest Statement:

The authors declare that there are no conflicts of interest

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Legend to Figure: schematic representation of the described bone marrow perivascular cell populations controlling adult hematopoietic stem cell (HSC) activity.

Human bone marrow is located within the trabecular region of the large bones, highly vascularised and rich in microvessels and capillaries. Different subtypes of pericytes were described to support HSCs in the bone marrow. Pericyte subsets from independent studies are shown separately. This representation does not imply that these subsets are necessarily distinct, some being shown to overlap (see Table). In human bone marrow, **(A)** niche supportive $CD146^+$ pericytes expressing CXCL12, JAGGED-1, ANGPT-1 and SCF are present surrounding sinusoids. In murine bone marrow, **(B)** quiescent HSCs can be located close to arterioles and are regulated by Nes^+ and $Nes^{peri}NG2^+$ pericyte subset; **(C)** CXCL12-abundant reticular (CAR) cells, located around sinusoids promote HSC activation and express CXCL12, JAGGED-1 and SCF; **(D)** perisinusoidal Leptin Receptor ($LepR^+$) and Nes^+ pericytes promote HSC quiescence and express SCF and CXCL12 respectively. Only tested protein expression is shown.